APPENDIX B

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Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity

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Abstract ***Bacillus thuringiensis*** **Cry1Ac δ-endotoxin specifically binds a 115-kDa aminopeptidase-N purified from *Manduca sexta* midgut. Cry1Ac domain III mutations were constructed around a putative sugar-binding pocket and binding to purified aminopeptidase-N and brush border membrane vesicles (BBMV) was compared to toxicity. Q509A, R511A, Y513A, and 509–511 (QNR-AAA) eliminated aminopeptidase-N binding and reduced binding to BBMV. However, toxicity decreased no more than two-fold, indicating activity is not directly correlated with aminopeptidase-N binding. Analysis of toxin binding to aminopeptidase-N in *M. sexta* is therefore insufficient for predicting toxicity. Mutants retained binding, however, to another BBMV site, suggesting alternative receptors may compensate in vivo.**

Key words: Aminopeptidase-N; Brush border membrane vesicle; Surface plasmon resonance; *N*-Acetylgalactosamine; *Bacillus thuringiensis*

1. Introduction

Bacillus thuringiensis (Bt) produces insecticidal crystal proteins during its sporulation process. The crystal inclusions are solubilized in the insect midgut to produce protoxin which is further digested to an active toxin fragment by midgut proteases. The activated toxin binds to specific receptors on the surface of the midgut epithelial cell membrane. Membrane-bound toxin is believed to be inserted into the lipid membrane and form pores or ion channels, leading to insect death. The receptor-binding step is considered to be critical to its mode of action and in resistance development.

Receptor-binding properties have been examined with brush border membrane vesicles (BBMV) prepared from the insect larval midguts and labeled Bt toxins [1]. Recently, surface plasmon resonance (SPR) techniques have been employed as well to study interactions between toxins and purified receptors [2–6]. Aminopeptidase-N (APN) was first identified [7] and purified [8] as a Cry1Ac receptor from *Manduca sexta* BBMV. APN from other insects were also identified as Cry1Ac receptors [9–11]. Cry1Ac binding to APN is inhibited specifically by *N*-acetylgalactosamine (GalNAc) [2,3,12], providing evidence that a carbohydrate moiety on APN is involved in toxin binding. A 210-kDa cadherin-like Cry1A-binding protein from *M. sexta* has also been reported [13,14].

The X-ray crystal structure of Cry1Aa reveals a three-domain composition [15]. Domain I, composed of seven α-helices, is important for membrane insertion and pore formation. Domain II, composed of antiparallel β-sheets, is involved in binding to BBMV and to purified receptors. Domain III, composed of a β-sheet sandwich, is also involved in receptor binding and ion-channel conductivity. Domain III shares the typical jelly roll topology found in other sugar-binding proteins that often lack primary sequence homology. Similar folds can be seen in sugar-binding bacterial toxins, viral proteins, glycosidases, and plant, animal, and fungal lectins. Lectins, however, have more than one sugar-binding site, and thus, can agglutinate cells [16]. Due to its abundance in intestinal tracts [17], many pathogenic lectins appear to have evolved to target GalNAc.

Previously, we have constructed alanine substitution mutations (residues 502–523) in the β-sheet in domain III of Cry1Ac toxin, and their biological activities and BBMV binding to several insects, including *M. sexta*, *Lymantria dispar*, *Heliothis virescens* were examined [18,19]. Mutations at the residues Q509, R511, and Y513 affected toxicity and BBMV binding to these insects. In the present paper, we have further examined the binding properties of these mutant toxins to *M. sexta* APN using SPR to evaluate the relationship between toxicity and purified receptor binding.

2. Materials and methods

2.1. Mutant toxin construction, expression, and purification

A putative 3D model of Cry1Ac was constructed by homology modeling from Cry1Aa [15] using SWISS-MODEL [20] and visualized using SWISS-PdbViewer v.3.1 with Q3D rendering. Site-directed mutagenesis of *cry1Ac1* [21] was carried out by the Kunkel method [22] and transformed into *Escherichia coli* MV1190 for expression. Active 65-kDa Cry1Ac toxins were produced as described previously [23]. Active proteins were further purified by size-exclusion chromatography on a Superdex 200 column (Amersham Pharmacia Biotech). Monomeric fractions were collected, and MW verified by dynamic light scattering (DynaPro-801). The monomeric form of the toxin was not found to multimerize in storage once column-purified from aggregate toxin forms. Mutants were analyzed by 10% SDS-PAGE and shown to be equal in size with wild type after trypsinization and size-exclusion purification.

2.2. BBMV preparation and binding studies

M. sexta BBMV were prepared by the magnesium precipitation method [24]. Iodination of toxins, BBMV competition binding, and ligand blotting were conducted as described previously [23,25].

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2.3. SPR analysis of binding to purified M. sexta APN

M. sexta APN was purified and tested for Cry1Ac binding ability as described in [6]. SPR technique was carried out using a BIAcore 2000 (Biacore AB, Uppsala, Sweden) with a CM5 sensor chip. All toxins were dialyzed into HBS (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA pH 7.4). Amine coupling of the soluble, 115-kDa Cry1Ac-binding APN was achieved in ammonium acetate, pH 4.2. Five randomized toxin concentrations (10–1000 nM) were 'kinjected' (low dispersion injection for kinetic measurements) at 30 μl/min. The experiment was repeated on three different APN-containing flow cells with two independent APN preparations. APN was regenerated by two 15–30-μl pulses of 10 mM NaOH, pH 11.0. Wild-type Cry1Ac binding was checked before and after all mutants to ensure the integrity of APN. Fittings were done globally using BIAevaluation 3.0. All models available with the software were tested, and the heterogeneity model (T+R1⇒TR1, T+R2⇒TR2) was chosen as the best fit ($\chi^2 < 1$). Initial global values for k_{on} and k_{off} were obtained from local fittings. Standard error is < 10% of the reported value for all apparent rate constants.

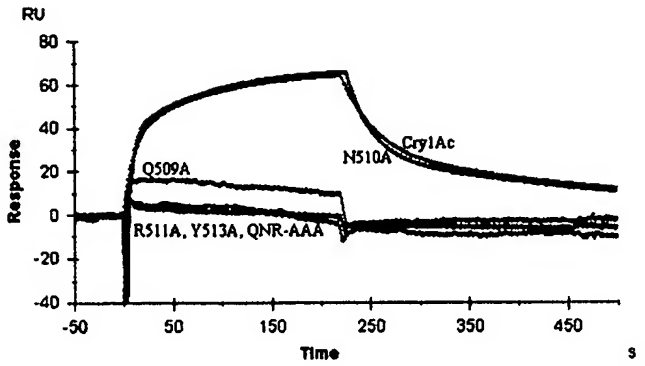

Fig. 2. Sensorgram overlay comparing wild-type Cry1Ac and mutant toxin binding (100 nM) to purified *M. sexta* APN.

3. Results

3.1. Cry1Ac domain III mutants and biological activities to M. sexta

Alanine substitutions were made in *cry1Ac1* at Q509, N510, R511, Y513, and at residues ^{509}QNR^{511}. When expressed, all mutants formed toxins that were stable after trypsin activation and analyzed by 10% SDS-PAGE (not shown). CD spectra of the mutant toxins were comparable to that of wild-type Cry1Ac, suggesting that the mutations do not alter structural integrity [19]. In sequence alignments with *cry1Aa* and *cry1Ab*, these residues are found in a non-conserved loop region extending from β-15 and continuing into β-16. A 3D structure of Cry1Ac was homology-modeled from Cry1Aa. Where a relatively flat surface is displayed on Cry1Aa, a cavity is predicted to form on the surface of Cry1Ac domain III. The mutants presented in this study line the bottom of the cavity (Fig. 1). Bioassays revealed that the mutant toxins are about two-fold less toxic than the wild-type Cry1Ac toxin (LC_{50} = 4.7 ng/cm^2). The range of LC_{50} values for the mutant toxins is 7.5–9.8 ng/cm^2. Detailed bioassay data are presented elsewhere [19].

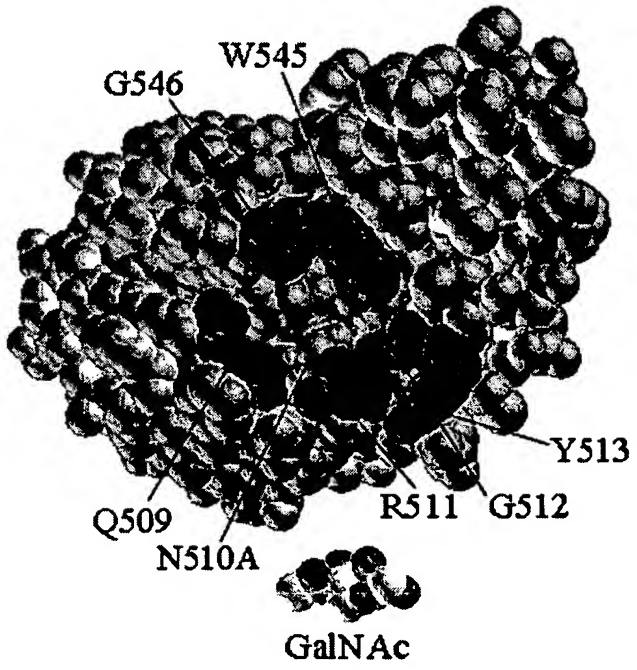

Fig. 1. Molecular model of Cry1Ac domain III GalNAc-binding pocket. Residues lining the cavity mouth are in black. GalNAc structure is shown for size comparison.

3.2. Real-time binding to purified APN

A BIAcore 2000 enabled the use of the SPR technique to monitor changes in mass on a surface of immobilized receptor as toxin is injected. Apparent rate constants were obtained by global analysis using BIAevaluation 3.0. Monomeric Cry1Ac bound *M. sexta* APN heterogeneously (Fig. 2) (T+R1⇒TR1, T+R2⇒TR2), confirming previously published results [2]. Wild-type apparent rate constants obtained were $k_{on1} = 3e^5$ M^{-1} s^{-1}, $k_{off1} = 3e^{-2}$ s^{-1} and $k_{on2} = 4.4e^4$ M^{-1} s^{-1}, $k_{off2} = 3.4e^{-3}$ s^{-1}, yielding K_D affinities of 141 nM and 77 nM, respectively. N510A bound APN with wild-type affinity (apparent rate constants were within standard error range of wild-type). No binding to APN was seen for Q509, R511, Y513, or QNR-AAA at any concentration. Therefore, no rate constants were measured for these mutant toxins.

3.3. BBMV receptor-toxin overlay

M. sexta BBMV proteins were separated by 6% SDS-

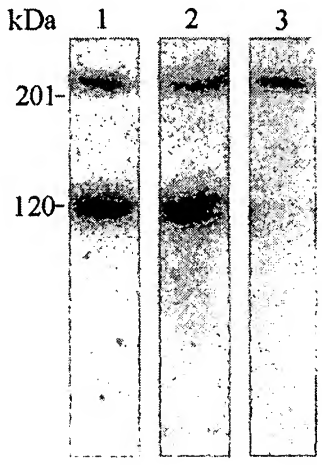

Fig. 3. Binding of ^{125}I-labeled Cry1Ac (lane 1), N510A (lane 2), and QNR-AAA toxin (lane 3) to *M. sexta* BBMV proteins. BBMV proteins (20 μg) were separated by 6% SDS-PAGE, transferred to PVDF, and probed with labeled toxins.

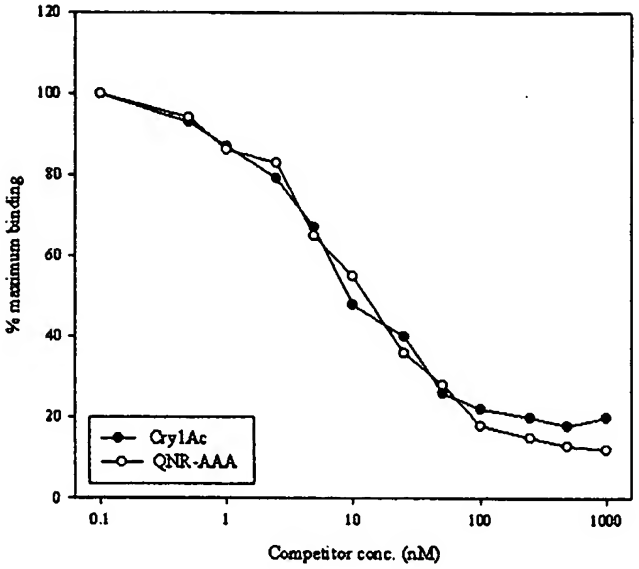

Fig. 4. Binding of Cry1Ac and domain III mutant toxins to *M. sexta* BBMV. ^{125}I-QNR-AAA (2 nM) was incubated with 10 μg BBMV in the presence of increasing concentrations of unlabeled Cry1Ac (●) or unlabeled QNR-AAA (○).

PAGE, transferred to PVDF membrane, and probed with ^{125}I-labeled toxins. Cry1Ac bound to a band slightly above 200 kDa, as well as a 120-kDa band, the molecular size of APN (Fig. 3). These results are in agreement with earlier findings for Cry1Ac [26]. N510A showed similar binding to wild-type. Conversely, labeled QNR-AAA showed no visible binding within the APN size range, but did retain binding to the higher band.

3.4. Binding to BBMV

In previous BBMV competition assays, we have shown that QNR-AAA and individual mutant toxins Q509A, R511A, and Y513A did not compete as well with labeled Cry1Ac for binding to BBMV compared to wild-type self-competition [19]. The affinity of Cry1Ac and QNR-AAA for *M. sexta* BBMV receptor sites was 3.9 (±1.2) and 38.2 (±5.3) nM respectively, reflecting a 10-fold difference. To further investigate the nature of QNR-AAA's reduced affinity, a reciprocal competition binding assay was conducted. Labeled QNR-AAA was competed with cold QNR-AAA or cold wild-type (Fig. 4). Cold Cry1Ac competes with labeled QNR-AAA with the same affinity that cold QNR-AAA does. The combined results of the reciprocal binding studies do not support the hypothesis that the QNR-AAA mutant toxin has reduced affinity for only APN. Rather, they suggest that Cry1Ac binds to multiple receptor sites, and that the QNR-AAA mutant toxin loses binding to APN sites while retaining binding to others.

4. Discussion

In the present study, a functional role for domain III of Cry1Ac was investigated by a series of mutations in a β-sheet region particular to Cry1Ac. Our results show considerable differences between binding affinities of wild-type and mutant toxin to *M. sexta* APN compared to differences in toxicity. The requirement of domain III residues for binding to *M. sexta* APN correlates with the findings in studies using domain-switched mutants [6,27,28] and ligand overlay blots using *M. sexta* BBMV [29]. Additionally, the loss of binding by domain III mutants to *Lymantria dispar* and *Heliothis virescens* BBMV [19] suggests these amino acids have a common role in binding to insect APNs. This idea is supported by studies that showed GalNAc inhibits Cry1Ac binding to both *L. dispar* and *H. virescens* APNs using the SPR technique [3,4]

A molecular model of Cry1Ac predicted these mutations to be located around a unique cavity that is not present on Cry1Aa. Residues W545 and G546 complete the upper portion of the cavity mouth (Fig. 1). N510 appears inside, and not surrounding, the cavity. The lip surrounding this indentation presents residues in Cry1Ac entirely non-conserved with Cry1Aa. Furthermore, the Cry1Aa amino acids 'filling in' the cavity from β-sheet 22 (S581, V582, F583) are missing in Cry1Ac, allowing G584 and N585 to line the pocket unobtrusively. GalNAc is known to inhibit Cry1Ac binding to purified APN, but not Cry1Aa [2]. This model provides evidence of structural differences between Cry1Ac and other Cry1A toxins that may account for the sugar-binding specificity of Cry1Ac.

Our experiments provide evidence that demonstrates Cry1Ac has multiple receptor specificity, which is lost when domain III mutations are constructed around the putative cavity. SPR studies and BBMV ligand blots showed QNR-AAA lost binding to APN from *M. sexta* BBMV, suggesting the unique cavity in Cry1Ac domain III is necessary for APN binding. However, QNR-AAA retained binding to another Cry1Ac-binding band that is also bound by wild-type. The presence of at least one other Cry1Ac-binding component in BBMV indicates that APN does not act as a lone receptor. Additionally, despite QNR-AAA's 10-fold loss of affinity for *M. sexta* BBMV in competition assays, it remains an equal competitor as wild-type for its binding to other receptor sites (Fig. 4). Therefore, the Cry1Ac mechanism of binding to APN (by domain III-sugar contact) may be non-essential for binding to other sites. Evidence of GalNAc-independent binding mechanisms has been observed in BBMV prepared from the anterior subregion of *M. sexta* midgut [30]. Furthermore, a report using various ligand binding protocols [26] found that a 210-kDa cadherin-like glycoprotein, BT-R1, from *M. sexta* binds Cry1A proteins. These data were strongly supported by the fact that cloned BT-R1 expressed in insect cells still bound Cry1A toxins [31]. A similar cadherin-like glycoprotein, BtR175, has been cloned from *Bombyx mori* that binds Cry1Aa [32] in a GalNAc-independent manner. Antibodies to BtR175 block insecticidal action in vivo [33].

Given the binding and toxicity contradiction found in this study, one possible explanation of our data is that while receptor recognition did not occur in in vitro binding studies, over the time course of a bioassay (5 days), only minor reductions in insecticidal activity occur. Alternatively, the first instar larvae used in bioassays and fifth instar larvae used for BBMV preparations may differ in a way that causes loss of binding at a later stage but not an earlier one. However, mutations in the domain II of several Cry toxins have been shown to reduce both toxicity and binding to BBMV in numerous publications. A second model to explain the contradiction is that Cry1Ac binding to another receptor is more functionally critical than binding to APN receptor. However, if APN acts as a binding protein without allowing the pore-

formation step, it should be considered a sequestering protein. In this model, mutants that lose binding to APN should be free to bind functional sites, thus augmenting toxicity. This is not the result of our bioassays. About two-fold less activity was observed for these mutant toxins. For APN to be non-critical in the gut environment it would have to be present in significantly lower ratios than other receptors, making loss of binding irrelevant. Neither BBMV ligand blots (Fig. 3) nor gut enzyme studies suggest a paucity of APN in insect guts. Furthermore, APNs from *M. sexta* and *H. virescens* have been reconstituted into membranes and caused toxin-induced pores to form [34]. In a final possible scenario, APN serves as a functional receptor among others. Although the binding to APN was completely abolished, binding to other receptors could be increased, which, in turn, maintain the toxicity over the time course of a bioassay. In this model, a mutant with enhanced affinity for APN might still enhance toxicity.

We note a recent report published while this paper was in preparation. The authors found similar Cry1Ac domain III mutations had no effect on toxicity to *M. sexta* [29]. The mutant toxins had reduced affinity for *M. sexta* BBMV as well as ability to induce BBMV permeability. Also, the mutants lost binding to the APN component of BBMV, as does Cry1Ac when competed with GalNAc. Here, we further advance a model for Cry1Ac receptor binding in this study by demonstrating a total loss of mutant toxin binding to purified APN using real-time kinetics. We see binding to other receptor sites by ligand blotting and reciprocal competition binding assays, which might account for the small changes in toxicity. Finally, we show a structural difference between Cry1Ac and Cry1Aa, which explains why Cry1Ac has sugar-binding specificity.

Mutations where loss of binding to purified APN is well correlated with loss of toxicity have been observed in Cry1Ac and Cry1Ab when domain II loop residues were altered (J. Jenkins and D. Dean, unpublished). A comprehensive binding model of domain III and domain II is under investigation to better understand the relationship between receptor binding and insecticidal activity.

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